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Lack of effect of 94 GHz radio frequency radiation exposure in an animal model of skin carcinogenesis

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Although there is no evidence that electromagnetic energy in the radio frequency radiation (RFR) band is mutagenic, there have been suggestions that RFR energy might serve as either a promoter or co-promoter in some animal models of carcinogenesis. Recent developments in electromagnetic technology have resulted in the manufacture of RFR sources capable of generating frequencies in the millimeter wavelength (MMW) range (30–300 GHz). Because absorption of MMW energy occurs in the skin, it is to be expected that long-term detrimental health effects, if any, would most likely be manifest in the skin. In this study we investigated whether a single (1.0 W/cm^2 for 10 s) or repeated (2 exposures/week for 12 weeks, 333 mW/cm^2 for 10 s) exposure to 94 GHz RFR serves as a promoter or co-promoter in the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced SENCAR mouse model of skin carcinogenesis. Neither paradigm of MMW exposure significantly affected papilloma development, as evidenced by a lack of effect on tumor incidence and multiplicity. There was also no evidence that MMW exposure served as a co-promoter in DMBA-induced animals repeatedly treated with 12-*O*-tetradecanoylphorbol 13-acetate. Therefore, we conclude that exposure to 94 GHz RFR under these conditions does not promote or co-promote papilloma development in this animal model of skin carcinogenesis.

Introduction

The radio frequency region of the electromagnetic spectrum is generally defined as including electromagnetic waves with frequencies in the range 3 kHz to 300 GHz. The controversy over whether radio frequency radiation (RFR) might initiate or promote cancer continues to receive a great deal of attention, both in the popular press and in the biomedical literature (reviewed in ref. 1). It is now well-accepted that RFR *per se* cannot be mutagenic, as the energy is insufficient to break chemical bonds (2–5). Studies investigating the carcinogenic potential of RFR have thus focused on whether it is a promoter or a co-promoter (defined as an agent that has no direct tumor-promoting activity but synergizes with a promoter to enhance tumor development). Several studies have investigated potential carcinogenic effects of RFR frequencies ranging from 435 to 2450 MHz on mammary cancer (6–9), liver cancer (10), lymphoma (11), brain cancer (12–15), colon cancer (16), sarcoma (17) and skin cancer (9,18,19). Additionally, exposures to ultra-wideband radiation have been performed in a murine model of mammary cancer (20).

As stated above, only three studies have investigated carcinogenic effects of RFR in models of skin cancer. In two of these studies (performed in the same laboratory), exposure to 2450 MHz RFR, either prior to or during initiation and promotion with 3,4-benzopyrene, accelerated the development of skin cancer and, consequently, decreased animal survival time (9,19). It was also noted, however, that the same acceleration of skin cancer development and reduction in survival occurred in animals exposed to chronic confinement stress in the absence of RFR exposure, suggesting that the hypothesized RFR effect could possibly be due to a non-specific stress reaction (9). In the third study, chronic exposure to 2450 MHz RFR did not alter tumor development in a mouse model of melanoma (18).

Recently, hardware systems capable of generating RFR of millimeter wavelength (MMW) (30–300 GHz) have been developed and are increasingly being used for a number of applications, such as satellite communications, military radar, non-lethal weapons, high speed data communications, automotive anti-collision devices, weapons detection and medical devices. To date, no study has investigated possible carcinogenic effects of MMW frequencies. The depth of penetration of 94 GHz RFR has been calculated to be ~0.3 mm (21–24), suggesting that all of the MMW energy absorption in animals and humans will occur in the skin. It is therefore most likely that, if any long-term carcinogenic effects exist, they would be observed in the skin. If such effects exist, it is also probable that MMW exposure would promote rather than initiate carcinogenesis. Hence, the goal of this study was to determine whether a single or repeated exposure to 94 GHz RFR serves as either a promoter or a co-promoter in the development of skin cancer.

To investigate this question, we used a two-stage model of carcinogenesis that utilizes SENCAR mice initiated with dimethylbenz[*a*]anthracene (DMBA) and promoted by repetitive application of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to elicit skin papillomas (25). A papilloma is a benign epithelial neoplasm consisting of villous or arborescent outgrowths of fibrovascular stroma covered by neoplastic cells; although benign, papillomas are premalignant lesions that will progress to malignant squamous cell carcinomas with extended observation times (25,26). The SENCAR mouse model has been extensively used to study carcinogenic effects of 60 Hz magnetic fields (27–32) and is therefore an established model

Abbreviations: ANOVA, analysis of variance; BrdUrd, 5-bromodeoxyuridine; DMBA, dimethylbenz[*a*]anthracene; ID, interior diameter; IR, infra-red; MMW, millimeter wavelength; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline; RFR, radio frequency radiation; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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for the study of potential effects of non-ionizing radiation on skin carcinogenesis.

Because MMW exposure at the levels investigated herein produce hyperthermia, it was necessary to delineate whether any effects noted were specific to MMW exposure or if they were simply a result of the hyperthermia attendant on MMW exposure. In order to examine this question, the biological effect of MMW exposure was compared with comparable thermal levels of infra-red (IR) exposure, which has a penetration depth similar to that of 94 GHz RFR (21,23). Conflicting reports appear in the literature, suggesting that hyperthermia treatment (via a water bath) can either serve as an anti-promoter (33,34) or as a promoter (35), depending on the treatment regimen.

Materials and methods

Animal procurement and quality assurance

Female SENCAR mice (4–5 weeks of age upon receipt, 30 g body wt) were obtained from the National Cancer Institute (Frederick, MD). Upon arrival at Brooks Air Force Base, 5% of the animal shipment was randomly removed for quality control testing, which included necropsies and microscopic examinations. A 10 day animal quarantine and a review of test results on each animal lot showed no indication of disease or parasites. Upon release from quarantine, animals were delivered to the housing facility where they were maintained for the duration of the experiment.

Animal housing facility

Each room in the animal housing facility was equipped with independent temperature, humidity, ventilation and light controls. Room temperature was maintained at 24 ± 1°C, with relative humidity at 50 ± 5%. Air flow rate was constant at 18 exchanges/h. A time controlled lighting system provided a 12 h light/12 h dark cycle (lights on at 0600). During most of the experiments the illumination level was no greater than 279.86 lux at any cage level. During the initiation phase of each experiment (see below) 'light covers' were utilized to allow an illumination level of no greater than 161.46 lux at any cage level and 43.06 lux at 1.0 m above the floor. Throughout all experiments the location of each animal cage on the racks was systematically changed weekly to ensure that the environmental conditions, including illumination levels, were similar for all mice. The experimental rooms (used for chemical application and MMW/IR exposures) were adjacent to the animal housing facility to minimize stress and environmental contamination during transport of the animals.

Animal husbandry

Animals were housed in groups of up to 7 per cage in Plexiglas cages (41×21×20 cm) with stainless steel wire covers and were allowed *ad libitum* access to food and water. The mice were provided with Lab Diet 5015 mouse diet (PMI International, Brentwood, MO) that contained only 4.5% of its calories as fat. Corncob bedding (1/8 inch; Green Pet Products, Conrad, IA) was used throughout each study and clean cages and fresh water were supplied to the animals twice a week. During the experimental period all animals were visually inspected twice daily (Monday–Friday); once between 0730 and 0830 and again between 1530 and 1630. On weekends animals were inspected once daily.

Papilloma initiation

At 7 weeks of age all animals were anesthetized with Metofane (methoxyflurane, catalog no. 55685; Pitman-Moore, Mundelein, IL), permanently marked for identification using a toe clip method (36) and the dorsoscapular region of the back (~2.5×4 cm) was shaved using multiple electric shavers to avoid overheating of the skin. On the following day mice were examined and excluded from the experimental groups (see below) if: (i) dermal abrasions, nicks or cuts occurred during shaving; (ii) general health or skin problems were evident; (iii) hair re-growth occurred within 24 h of shaving; (iv) mice were outside the weight range.

Two days following shaving and toe clipping, animals were initiated by treatment with a topical application of DMBA (catalog no. 1176775; Fisher Scientific, Houston, TX). The DMBA was mixed on the day of initiation and applied at a dose of 10 nmol in 200 µl of acetone to the shaved dorsoscapular region of the back. DMBA initiation occurred under subdued lighting conditions within the laboratory, as DMBA is light sensitive. Following DMBA initiation, mice were housed in disposable cages (4 mice/cage, 25×13×12 cm; Lab Products, Seaford, DE) with filter tops for 2 weeks in order to eliminate residual DMBA that might have been transferred to the cages or bedding. During these 2 weeks food and water were provided *ad libitum* and clean cages were supplied twice weekly. These initiated animals were maintained under low intensity yellow light conditions (yellow filter no. 4VC50; Grainger, San Antonio, TX). Following this 'wash-out' period animals were returned to conventional Plexiglas cages and treatment of animals was continued according to the experimental design (see below). We arbitrarily designated week 1 as the first full week after the last day of the DMBA 'wash-out' period.

When appropriate to the experimental design, control animals were included that were not initiated with DMBA. These control animals received only an application of 200 µl of the vehicle (acetone). Dosing of animals with acetone was performed under identical environmental conditions (i.e. subdued lighting) to those used for DMBA dosing. Following this vehicle dosing animals were housed and handled exactly as the DMBA-treated animals.

Papilloma promotion protocol

Animals received a specified dose of TPA (catalog no. 445004; Alexis Biochemicals, San Diego, CA), administered in 200 µl of acetone, applied to the dorsoscapular region of the back. All TPA promotion occurred twice a week between the hours of 0800 and 1200. Control animals received 200 µl of acetone (TPA vehicle).

The TPA stock solution (838 µg/ml TPA) was mixed at the beginning of each experimental series and was stored in an amber colored glass bottle at –20°C. TPA was diluted in acetone from the stock solution on each day of application.

Exposure systems and dosimetry

MMW exposure system

Animals were exposed in the far field to 94 GHz RFR (continuous wave) (Applied Electromagnetics, Marietta, GA). A dielectric lens was placed 88 cm from the conical transmitter horn (24 mm i.d.) to increase the power density and focus the beam (37). Since the resulting field was parallel to the floor, a 45° aluminum reflector was placed 120 cm from the lens to aim the MMW field towards the animal's dorsal surface. The animal was located 60 cm below the aluminum reflector. In preliminary experiments IR thermography (FLIR Systems, Portland, OR) was used to determine the increase in skin temperature elicited by MMW or IR exposure at various power densities; the power densities chosen for use in the actual experiments were those required to elicit an increase in skin temperature of 13–15 and 4–5°C in Experiments 1 and 2, respectively. In addition, IR thermography was performed during Experiment 2 at the beginning of the co-promotional phase (week 1) and at the end of the co-promotional phase (after week 12) to ensure that the increases in skin temperature produced by either MMW or IR exposure were consistent throughout the experiment.

IR exposure equipment

Two 250 W IR heat lamps (1100 nm peak spectral irradiance, catalog no. 250R40/10; Osram Sylvania Products, St Mary's, PA) were mounted 30 cm above the exposure area. The combined field intensity emitted from both lamps was adjusted using a Via-Stat Power Transformer (Superior Electric Co., Bristol, CN). Field intensity of both lamps was calibrated with a radiometer (Molectron Detector, Portland, OR) located at the center of the exposure area.

Restrainers

Each animal was exposed or sham exposed in a custom-designed Styrofoam restrainer that ensured correct placement of the animal during exposures to MMW and IR (38). A rectangular region (2.25×3 cm) was removed from the top part of the restrainer to permit exposure of only the dorsoscapular region of the mouse. Each restrainer held only one animal and each animal was exposed individually.

Experiment 1 (single exposure)

The goal of this experiment was to determine whether a single dose of MMW exposure (1.0 W/cm^2 for 10 s) was sufficient to promote skin cancer in DMBA-initiated SENCAR mice. All animals in this experiment were initiated with DMBA. Following the end of the DMBA 'wash-out' period, animals with a body weight that varied more than 20% from the mean of the entire colony were excluded from the experimental groups. All remaining animals were randomly assigned to one of the three experimental groups (MMW, IR and sham, n = 55/group) using body weight as a blocking variable to ensure that there were no statistical differences in initial group mean body weights.

In a pilot study IR thermography was used to determine the rise in skin temperature elicited by MMW exposure (1.0 W/cm^2 for 10 s). The IR exposure parameters required to elicit the same increase in skin temperature (13–15°C) were determined and subsequently used throughout the experiment.

During the experiment each mouse was anesthetized (Metofane), placed in a restrainer and the dorsoscapular region of the back was exposed once to MMW (1.0 W/cm^2 for 10 s) or IR (1.5 W/cm^2 for 10 s) or was sham exposed

(placed in a restrainer for 10 s without irradiation). After exposure all animals were returned to their home cages and allowed to regain consciousness.

In addition to the three experimental groups, a positive control group (n = 27) was included to ensure the success of chemical initiation by DMBA. This positive control group was initiated at the same time as the experimental animals, using the same mixture of DMBA, however, this group was not exposed to MMW or IR, but received promotion by TPA (3.4 nmol in 200 µl acetone) twice weekly for 23 weeks.

For the duration of the experiment (23 weeks) each animal was weighed, visually inspected and palpated once each week for papilloma formation. The individuals performing the visual inspection and palpation of the animals were blind as to the animals assigned to each experimental group. At the end of the 23 week period mice were killed by CO_2 inhalation. Five animals were randomly chosen from each group for necropsy. Any other animals displaying irregularities of the skin (i.e. lesions that appeared to be abnormal papillomas) were also selected for necropsy and examined via histopathology.

Experiment 2 (repeated exposure)

The goal of this experiment was to determine if repeated MMW exposure (2 exposures/week for 12 weeks, 333 mW/cm^2) was sufficient to promote or co-promote skin cancer in DMBA-initiated SENCAR mice.

A pilot study was performed in order to determine the optimal dose of TPA to be used as the co-promoter. In this pilot study all animals were toe clipped and shaved as described above. Before DMBA initiation took place animals were separated into three experimental groups (n = 35/group) using body weight as a blocking variable. Groups 1 and 2 were initiated with DMBA (10 nmol in 200 µl acetone) while group 3 was treated with vehicle (acetone). Beginning 2 weeks following DMBA initiation and continuing for 16 weeks, group 1 received 0.85 nmol TPA and groups 2 and 3 received 1.7 nmol TPA. As described in the promotion protocol, all animals received TPA twice weekly on Tuesday and Friday between the hours of 0800 and 1200. All dosing with TPA was performed according to experimental group, starting with group 1 (receiving 0.85 nmol TPA) and progressing to groups 2 and 3 (1.7 nmol TPA).

For the duration of the pilot study (16 weeks) experimental animals were weighed, visually inspected and palpated for papilloma formation once each week by individuals who were blind as to the animals assigned to each experimental group. At the end of the 16 week period mice were killed by CO_2 inhalation and animals were chosen for necropsy as described above.

The pilot study identified the TPA dose (0.85 nmol) to be used in the repeated exposure experiment (see Figure 1). This concentration of TPA reduced the possibility of a 'ceiling effect' occurring. New animals were shaved and toe clipped and then separated into eight groups (n = 50/group) using body weight as a blocking variable (see Table I). All groups except groups 3 and 8 were initiated with DMBA (10 nmol in 200 µl acetone); groups 3 and 8 served as control groups and were treated with 200 µl of acetone during the initiation phase.

Following the end of the 2 week DMBA 'wash-out' period animals were sham exposed or exposed to MMW or IR in combination with either TPA (0.85 nmol) (groups 2, 5 and 7, respectively) or acetone (groups 1, 4 and 6, respectively). The days for MMW, IR or sham exposures coincided with those for TPA application throughout the course of the co-promotional phase (first 12 weeks after the DMBA 'wash-out' period) of the experiment. In order to complete all experimental manipulations (i.e. exposures and TPA/vehicle applications) within the time period 0800–1200 h, each experimental group was randomly divided into two subgroups. One subgroup was exposed and promoted twice weekly on Monday and Thursday while the other subgroup was exposed/promoted on Tuesday and Friday. The designated treatment days for each group were kept constant during the full 23 week course of the experiment.

Before each exposure on Monday and Tuesday animals were weighed, visually inspected, and palpated. During each exposure animals were conscious and individually restrained and the dorsoscapular region of the back was either sham exposed (placed in a restrainer without irradiation for 10 s) or exposed to MMW (333 mW/cm^2 for 10 s) or IR (600 mW/cm^2 for 10 s). As before, the IR exposure parameters were chosen in order to match the increase in skin temperature (4–5°C) produced by the MMW exposure.

Following exposure animals were removed from the restrainer and immediately treated with either TPA or acetone. Application of the promoter or vehicle was performed after exposure to prevent any possible alteration in energy absorption due to the presence of fluid on the exposed region of the back. Animals were then returned to their home cages.

At the end of 12 weeks exposure of animals to MMW, IR or sham conditions was discontinued, however, TPA application continued until termination of the experiment at 23 weeks. Throughout the 23 week experimental period evaluators, blind as to which animals were assigned to each experimental group, counted and measured papillomas. At the end of the 23 week period mice were killed by CO_2 inhalation and necropsied as described in Experiment 1.

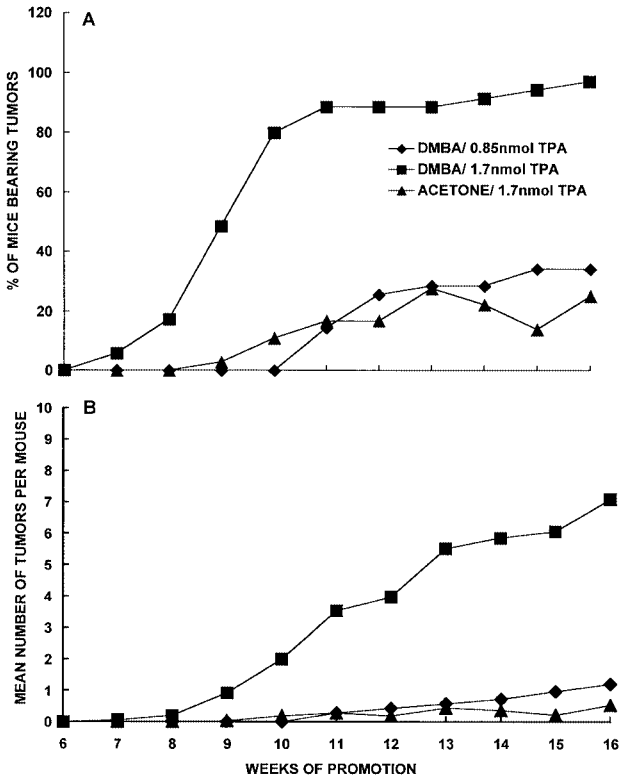


Fig. 1. Mean data from the pilot study in Experiment 2 showing tumor incidence (**A**) and multiplicity (**B**) data for SENCAR mice exposed to three experimental conditions (n = 35/group). Mice were initiated with DMBA or acetone (vehicle) and then promoted with TPA (0.85 or 1.7 nmol).

Table I. Groups in Experiments 2 and 3

Group	DMBA	Exposure condition	TPA treatment
1	+	Sham	–
2	+	Sham	+
3	–	Sham	+
4	+	MMW	–
5	+	MMW	+
6	+	IR	–
7	+	IR	+
8	–	Sham	–

In some groups, DMBA (10 nmol in 200 µl acetone) was administered to initiate papilloma formation. These animals were then sham exposed or exposed to MMW or IR in combination with either TPA (0.85 nmol) (groups 2, 5 and 7, respectively) or acetone (groups 1, 4 and 6, respectively). Groups 3 and 8 served as control groups and were treated with 200 µl of acetone during the initiation phase.

Experiment 3

This experiment determined whether the repeated MMW exposure conditions used in Experiment 2 would induce alterations in well-accepted short-term biomarkers of carcinogenesis (27). Phase 1 of this experiment examined epidermal thickness and incorporation of 5-bromodeoxyuridine (BrdUrd) into DNA, whereas Phase 2 examined epidermal ornithine decarboxylase (ODC) activity. ODC is a key enzyme in the biosynthesis of polyamines that promote cell proliferation and the transition of many mammalian tissues from a relatively quiescent state to a rapidly proliferating population of cells is accompanied by large increases in ODC levels (39). All animals were shaved and toe clipped as described previously. Before DMBA initiation took place animals were separated into eight experimental groups using body weight as

a blocking variable. Experimental groups and animal treatments were identical to those in Experiment 2 (see Table I).

Epidermal thickness and BrdUrd incorporation

Collection of skin samples. Four animals per experimental group were killed by CO_2 inhalation at the end of 1, 2 and 5 weeks of treatment. Animals were killed 48 h after the most recent TPA treatment (0.85 nmol). One hour before death BrdUrd (Ccatalog no. B5002; Sigma, St Louis, MO) was dissolved in Dulbecco's 1× phosphate-buffered saline (PBS) at a concentration of 20 mg/ml. Thirty minutes before death animals were injected i.p. with BrdUrd at a dose of 100 µg BrdUrd/g body wt. Immediately following death a depilatory (Nair) was applied to the dorsoscapular region of the back (for 30–60 s) and the hair was removed by gently scrubbing the back under cold water. Skin from the entire dorsoscapular area of the back was carefully excised and placed in 10% formalin. The excised skin was cut parallel to its long axis to yield two halves. One of these halves was cut parallel to its long axis into four 1 mm wide strips, each of which was fixed in 10% formalin for 12 h and then paraffin embedded. Paraffin blocks were sliced into 5 µm thick sections and the resulting slices were mounted on glass microscope slides.

Epidermal thickness: Skin sections were stained with hematoxylin and eosin. Epidermal thickness was evaluated using light microscopy by a board-certified veterinary pathologist who was blind as to the animals assigned to each experimental group. Epidermal thickness across the length of the strip that had been adjacent to the midline axis (i.e. the region above the spine) was evaluated on a scale from 0 to 4 according to the following criteria: 0, normal epithelium, ~1–3 cell layers thick with a thin stratum corneum; 1, minimal hyperplasia, epithelium ~4–6 cell layers thick; 2, minimal to mild hyperplasia, variable amounts of hyperplasia across the specimen, areas fitting each description present; 3, mild hyperplasia, epithelium ~7–9 cell layers thick or epithelial layer composed of ~4–6 hypertrophic cells; 4, moderate hyperplasia, epithelial layer ~10–15 cell layers thick.

BrdUrd incorporation: BrdUrd incorporation into DNA was detected using immunohistochemical techniques (40). Staining consisted of deparaffinizing the skin sections and then incubating sections in a 0.05% protease (P5380, Type 8; Sigma) solution for 20 min followed by a wash with 0.01 M PBS/albumin. Sections were incubated in a 1:1500 dilution of BrdUrd primary antibody (33281A; BD PharMingen, San Diego, CA) for 12 h at 4°C. Sections were then rinsed in 0.01 M PBS/albumin and incubated in biotinylated secondary antibody (mouse IgG, PK-4002; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After rinsing with 0.01 M PBS/albumin, sections were incubated in the Vectastain ABC mixture from the above PK-4002 kit for 30 min at room temperature. After rinsing with 0.01 M PBS, sections were stained with a 0.1% 3,3′-diaminobenzidine tetrahydrochloride (D5637; Sigma)/0.02% hydrogen peroxide solution for 2–3 min. Slides were soaked in 70% and 95% alcohol and 100% 2-propanol for 2 min each. Sections were then rinsed in AmeriClear (C4200-1; Baxter Scientific, McGaw Park, IL) and coverslipped using Permount (SP15-100; Fisher Scientific, Fair Lawn, NJ).

Using light microscopy, random interfollicular fields were selected for scoring of BrdUrd-labeled nuclei. A total of 500 basal cells (labeled and unlabeled) adjacent to the basement membrane were evaluated and the labeling index was expressed as percent labeled cells. The microscopist was blind as to which animals belonged in each experimental group.

Epidermal ODC activity

Four animals per experimental group were killed by CO_2 inhalation at the end of weeks 1, 2 and 5 of treatment. To serve as positive controls, additional mice were killed at 1 and 10 weeks of promotion with TPA (3.4 nmol). All animals were killed 6 h after the most recent TPA or acetone treatment. Immediately following death a depilatory (Nair) was applied to the dorsoscapular region of the back (for 30–60 s) and the hair was removed by gently scrubbing the back under cold water. The backs of the mice were kept on ice until the skin samples were collected. Skin from this dorsoscapular area of the back was carefully harvested and spread on a chilled glass plate. The epidermal layer was removed by scraping the skin with a razor blade. Scrapings from each mouse was placed directly into 0.5 ml of homogenizing buffer, quick frozen with liquid nitrogen and stored at –70°C.

To achieve detectable ODC activity levels, epidermal samples from two mice within a treatment group were mixed together to form a single assay sample. This resulted in two assay samples within each treatment group. Epidermal tissue samples were placed in homogenizing buffer (25 mM sodium phosphate buffer, 5 mM dithiothreitol, 0.05 mM EDTA, 0.1 mM pyridoxal 5′-phosphate). Samples were homogenized on ice using a Polytron homogenizer (PT 10/35) set on a high setting (15 s on/15 s off/15 s on, to minimize sample heating). The cell lysates were centrifuged at 12 000×for 30 min at 4°C. The clear lysates were transferred into fresh pre-chilled tubes. Total protein in 2 µl aliquots of each sample was measured by the bicinchonic acid method following the manufacturer's protocol (Pierce Chemical Co., Rockford, IL).

Samples were incubated in an assay mixture containing 35 mM sodium phosphate buffer, pH 7.2, 0.2 mM pyridoxal 5′-phosphate, 4 mM dithiothreitol, 1 mM EDTA and 0.4 mM L-ornithine (0.5 µCi [l-^{14}C]DL-ornithine hydrochloride). Samples were incubated at 37°C for 1 h in 15 ml Erlenmayer flasks equipped with rubber stoppers and center well assemblies. A 2:1 (v/v) mixture of ethanolamine and methoxyethanol (200 µl) was added to the center well assembly. After the incubation period the reaction was stopped by addition of 0.5 ml of 2 M citric acid. The flasks were further incubated for 1 h to ensure complete absorption of $^{14}CO_2$ by the ethanolamine and methoxyethanol mixture. Finally, the center well containing the ethanolamine/methoxyethanol mixture was carefully transferred to a vial containing 10 ml of toluene-based scintillation fluid and 2 ml of ethanol. Radioactivity was measured in a Beckman LS6000 SC liquid scintillation counter with >95% efficiency. The ODC enzyme activity was expressed as pmol $^{14}CO_2$ released/h/mg protein.

Statistics

Based on data from the published literature (27), the power calculations for Experiment 1 showed that with 50 animals/group there would be a 90% power to detect a significant change ($P \leqslant 0.05$) if the number of animals developing papillomas (tumor incidence) in each group differed by more than 8 (16%). Based on the TPA data from DiGiovanni *et al.* (27), power calculations for Experiment 2 showed that with 50 animals/group the smallest change required in tumor incidence to produce significance was 28%.

In Experiments 1 and 2 statistical analyses of tumor incidence at 16 and 23 weeks of promotion were performed using the χ^2 test (41). For multiplicity data mean number of tumors per mouse was calculated for each treatment group at each week of promotion. Mann–Whitney *U*-tests were used to compare treatment groups at 16 and 23 weeks of promotion. All analyses were performed using Statistica (StatSoft, Tulsa, OK). Statistical significance was considered to be $P \leqslant 0.05$.

In Experiment 3 the epidermal thickness and BruUrd-labeled cells were each evaluated using analysis of variance (ANOVA) with a Newman–Keuls *post hoc* test. The assay for epidermal ODC activity required the pooling of epidermal cells resulting in two assay samples per treatment group. Thus, there was no within statistical cell error term.

Results

Experiment 1 showed that a single dose of MMW exposure (1.0 W/cm^2 for 10 s) was insufficient to promote skin cancer in DMBA-initiated animals (see Figure 2). There were no significant differences in tumor incidence (percentage of mice bearing tumors) or multiplicity (mean number of tumors per mouse) in animals that were sham exposed or exposed to MMW or IR. TPA (3.4 nmol) promotion increased both tumor incidence and multiplicity.

Experiment 2 showed that repeated MMW exposure (twice weekly for 12 weeks, 333 mW/cm^2 for 10 s) was insufficient to promote or co-promote skin cancer in DMBA-initiated animals (see Figure 3). In the groups receiving vehicle (acetone) rather than TPA, the tumor incidence and multiplicity values were close to 0 and there were no significant differences in these parameters across experimental treatments. In the TPA co-promoted groups there were increased levels of tumor incidence and multiplicity as compared with groups that did not receive TPA co-promotion. However, the addition of repeated MMW or IR exposure did not further increase either tumor incidence or multiplicity in animals receiving TPA. The animals that were not initiated with DMBA but were promoted with TPA (Group 3) showed elevated levels of tumor incidence and multiplicity, but these levels were not as large as those of animals receiving both DMBA and TPA (Figure 3).

Experiment 3 evaluated the effects of repeated MMW exposure conditions on short-term biomarkers of carcinogenesis (epidermal thickness, BrdUrd incorporation into DNA and epidermal ODC activity). Epidermal thickness was essentially normal in animals receiving acetone rather than TPA and either sham exposed or exposed to MMW or IR (see

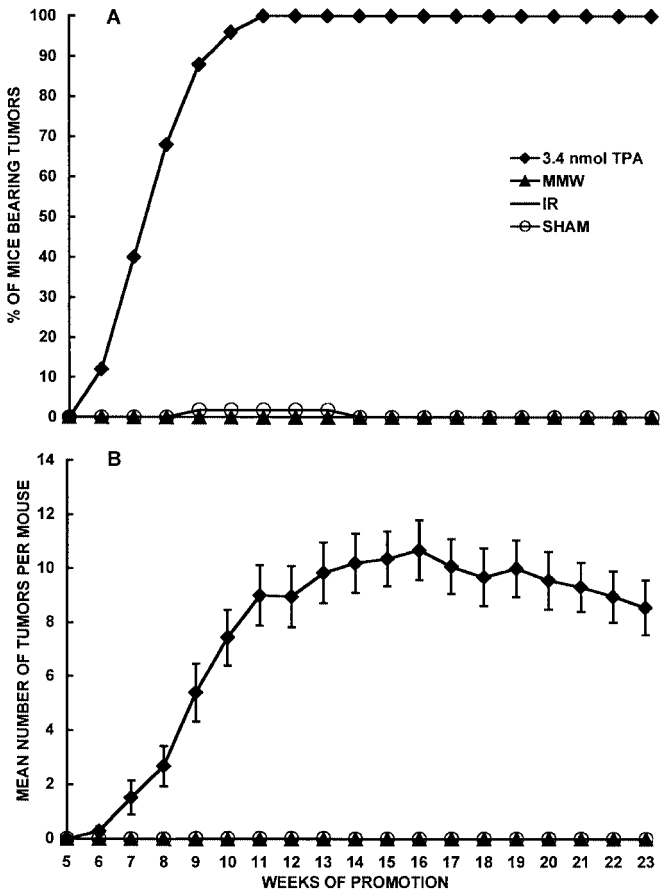


Fig. 2. Data from Experiment 1 showing tumor incidence (**A**) and multiplicity (**B**) data for SENCAR mice initiated with DMBA and then exposed once to MMW, IR or sham conditions (n = 55/group). In the MMW and IR conditions each mouse received a single 10 s exposure that raised local skin temperature 13–15°C. A positive control group of mice (n = 27) was promoted with TPA (3.4 nmol), but was not exposed to MMW or IR. Values shown are means ± SEM.

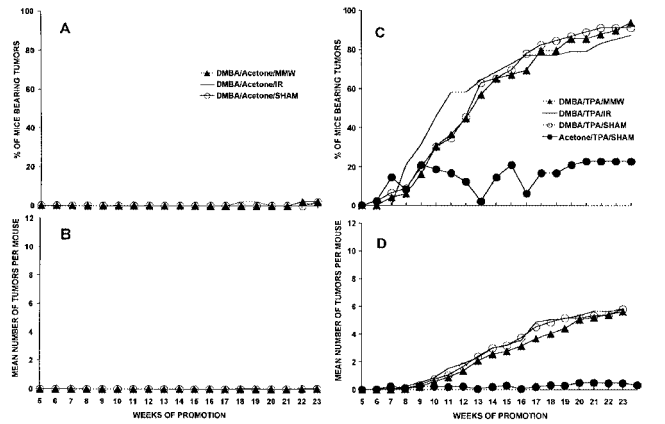


Fig. 3. Mean data from Experiment 2 showing tumor incidence (**A** and **C**) and multiplicity (**B** and **D**) data for SENCAR mice treated with DMBA or acetone and then treated with either acetone (vehicle) or TPA (0.85 nmol) (n = 55/group). In the MMW and IR conditions each mouse received repeated 10 s exposures (2 exposures/week for 12 weeks); each exposure raised the local skin temperature 4–5°C.

Figures 4A and 5B and C). Epidermal thickness in TPA-promoted animals increased substantially by week 1 and then gradually increased during the next 4 weeks. ANOVA revealed significant differences as a function of group ($P < 0.01$) and

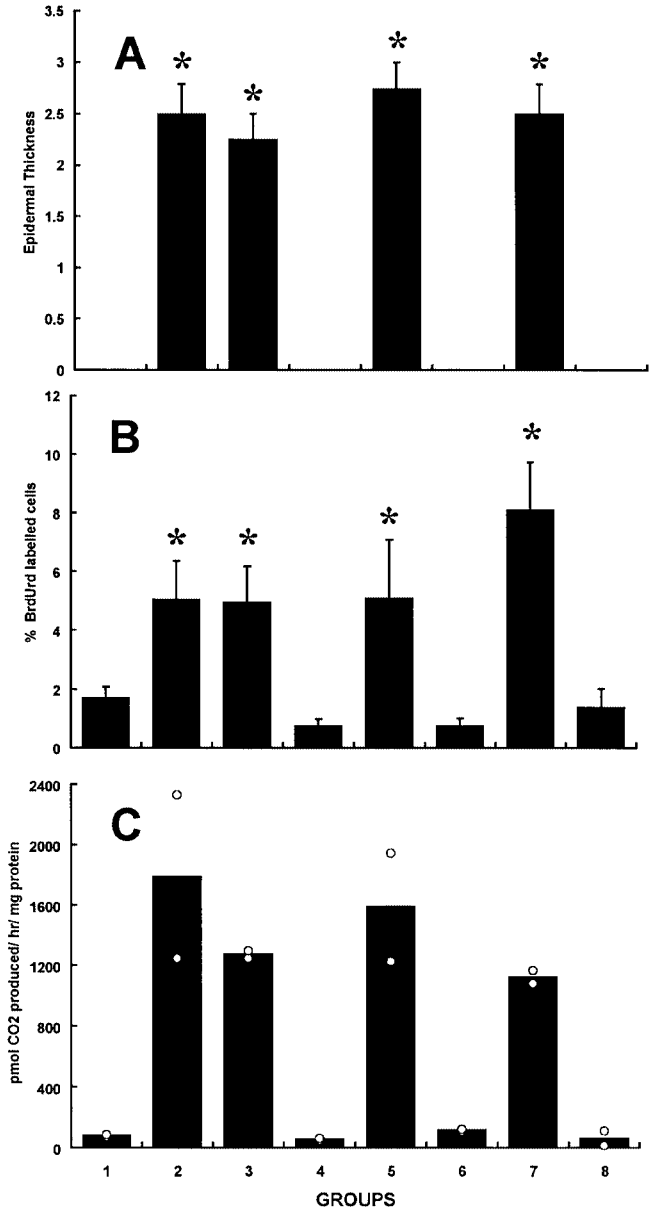
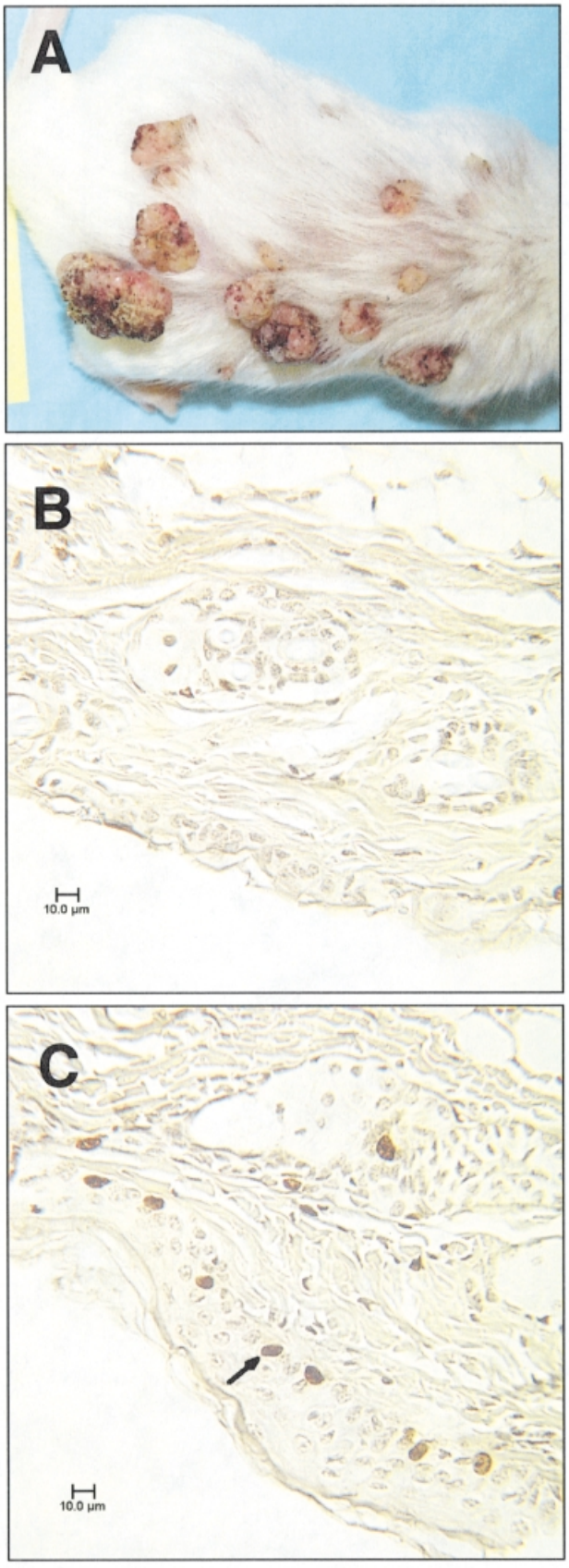


Fig. 4. (**A**) Epidermal thickness, (**B**) BrdUrd incorporation into DNA and (**C**) epidermal ODC activity (pmol CO_2 produced/h/mg protein) in SENCAR mice exposed to eight experimental conditions (n = 4/group) 5 weeks after the end of the DMBA 'wash-out' period. Group identification is as shown in Table I. Epidermal thickness was rated on a scale of 0–4: 0, normal epithelium, ~1–3 cell layers thick with a thin stratum corneum; 1, minimal hyperplasia, epithelium ~4–6 cell layers thick; 2, minimal to mild hyperplasia, variable amounts of hyperplasia across the specimen, areas fitting each description were present; 3, mild hyperplasia, epithelium either ~7–9 cell layers thick or epithelial layer composed of ~4–6 hypertrophic cells; 4, moderate hyperplasia, epithelial layer ~10–15 cell layers thick. BrdUrd incorporation into DNA was quantitated by evaluating 500 basal cells (labeled and unlabeled) adjacent to the basement membrane and the labeling index was expressed as percent labeled cells. In the MMW and IR conditions each mouse received repeated 10 s exposures (2 exposures/week for 12 weeks), each exposure raising the local skin temperature 4–5°C. The TPA concentration for groups 2, 3, 5 and 7 was 0.85 nmol in 200 µl of acetone. In (A) and (B) groups 2, 3, 5 and 7 were significantly different (*) from the non-TPA-promoted groups (groups 1, 4, 6 and 8). There were no significant differences among groups 2, 3, 5 and 7. Values shown are means ± SEM. Bars in (C) show mean epidermal ODC activity of two groups. Open circles show the actual values for each group.

weeks of TPA promotion ($P = 0.046$). The interaction term was not significant ($P = 0.339$). *Post hoc* analysis revealed that TPA promotion significantly increased epidermal thickness. There were no significant differences amongst TPA-treated animals that were sham exposed, exposed to MMW or exposed to IR.



There were few or no BrdUrd-labeled cells in animals receiving acetone rather than TPA and either sham exposed or exposed to MMW or IR (see Figures 4B, 5B and C). In TPA-promoted mice the number of BrdUrd-labeled cells increased substantially by week 1 and remained relatively constant during the next 4 weeks. ANOVA revealed a significant difference as a function of groups ($P < 0.01$), but not as a function of weeks ($P = 0.43$). The interaction term was not significant ($P = 0.63$). *Post hoc* analysis revealed that TPA promotion significantly increased the number of BrdUrd-labeled cells. There were no significant differences amongst TPA-treated animals that were sham exposed, exposed to MMW or exposed to IR. The number of BrdUrd-labeled cells in these TPA-treated animals was similar to that reported under similar experimental conditions by DiGiovanni *et al.* (27).

There was little ODC activity in the animals receiving vehicle (acetone) rather than TPA (see Figure 4C). Furthermore, there were no substantial differences among those animals that were sham exposed or exposed to MMW or IR. TPA promotion increased epidermal ODC activity gradually over the 5 week period. In these TPA-treated groups there were no substantial differences among animals sham exposed or exposed to MMW or IR.

Discussion

There are three principal conclusions derived from this study. First, a single MMW exposure (1.0 W/cm^2 for 10 s) that produces a 13–15°C increase in skin temperature does not promote the development of papillomas in DMBA-initiated SENCAR mice. Second, repeated MMW exposures (twice weekly for 12 weeks, 333 mW/cm^2) that produce a 4–5°C increase in skin temperature failed to either promote or act synergistically with TPA to co-promote the development of papillomas in this mouse model. Finally, repetitive exposure to MMW alone does not alter the expression of well-recognized early biomarkers and MMW exposure does not affect TPA-induced increases in these indices of epidermal hyperplasia.

To our knowledge, the present study is the first to investigate potential carcinogenic effects at frequencies in the MMW range. The experiments presented herein address two potential scenarios of accidental overexposure. The first is a single, accidental overexposure to a relatively high power density of MMW (Experiment 1). A single MMW exposure did not alter either tumor incidence or multiplicity in DMBA-initiated SENCAR mice, providing strong evidence that, under the conditions of the experiment, a single high dose of MMW is insufficient to promote tumor formation in previously initiated skin. This finding is not surprising, since even known promoting agents must be applied repeatedly to produce an increase in tumorigenesis in initiated skin (25). The second exposure scenario is that of repeated exposure to lower levels of MMW

Fig. 5. (**A**) Papilloma formation in a SENCAR mouse that was DMBA-initiated, sham exposed and promoted with TPA (0.85 nmol) for 21 weeks. Photomicrographs showing BrdUrd incorporation and epidermal thickness in skin from an animal that was (**B**) DMBA-initiated, sham exposed and treated with acetone for 5 weeks or (**C**) DMBA-initated, sham exposed and TPA (0.85 nmol) promoted for 5 weeks. The arrow points to a BrdUrd-labelled cell.

(Experiment 2). Again, MMW exposure (twice a week for 12 weeks) did not alter tumor incidence or multiplicity in DMBA-initiated mice, either alone or when MMW exposure was combined with treatment with a known chemical promoter (TPA). Thus, there was no evidence that MMW exposure under these experimental conditions served as either a promoter or a co-promoter in a well-accepted animal model of skin carcinogenesis (25).

This conclusion was further buttressed by the results from the assays of early biomarkers of tumor-promoting activity. Epidermal hyperplasia-associated effects were assessed using three different procedures: epidermal thickness (27), BrdUrd labeling index of epidermal cells (27,42) and epidermal ODC activity (39,43,44). Not surprisingly, all of these indices of epidermal hyperplasia were increased by promotion with TPA, as in previous studies (27,39,42–44). Exposure to MMW alone did not alter any of these early biomarkers and MMW exposure did not affect the increases in these indices of epidermal hyperplasia that were associated with chemical promotion by TPA. These results are consistent with the tumor multiplicity and incidence data and provide further support for the conclusion that MMW exposure does not promote tumorigenesis.

A number of similarities have been noted between skin cancer in humans and chemically induced skin cancer in SENCAR mice. As examples, the development of skin cancer in both species is associated with alterations in the tumor suppressor gene *p53* (45), loss of heterozygosity at specific chromosomal loci (46), altered cyclin kinase inhibitor activity (47,48) and increased clusterin protein expression during photodynamic therapy (49). Furthermore, skin tumorigenesis is inhibited by retinoids (50) and prevented by drugs that inhibit epidermal lipid peroxidation (51). Thus, the SENCAR mouse skin cancer model used in the present experiments is an appropriate model for extrapolation to questions concerning human skin cancer.

In a two-stage skin tumorigenesis protocol using DMBA-initiated SENCAR mice, Mitchel and colleagues demonstrated that hyperthermic treatment (44°C for 30 min in a water bath) could serve as a co-promoter if applied near the time of initiation (35), but as an anti-promoter if applied near the time of chemical promotion (33,34). Furthermore, although hyperthermia alone is not carcinogenic, hyperthermia may enhance the development of tumors induced by ionizing radiation (52,53). Based on this information, we included IR exposure in our experimental design to control for any non-specific effects of hyperthermia alone and to ensure that any carcinogenic effects of MMW exposure would be a product of the RFR energy rather than this potential confounding variable. The hyperthermia response to MMW or IR exposure did not alter tumor development under either experimental paradigm, despite the fact that hyperthermic exposure occurred in close time proximity to the application of TPA; additionally, neither MMW nor IR exposure significantly altered any of the early markers of tumorigenesis. The finding that hyperthermia did not serve as an anti-promotor in our experiments may be due to the relatively short exposure duration (10 s) as compared to the 30 min used by Mitchel and colleagues (33,34).

In summary, neither a single exposure to relatively high levels of 94 GHz RFR nor repeated exposure to lower doses of 94 GHz RFR altered either tumor incidence or multiplicity in DMBA-initiated SENCAR mice. Furthermore, repeated MMW exposures did not act synergistically with a known chemical promoter (TPA) to alter tumor incidence or multiplicity. Finally, repeated MMW exposure failed to alter epidermal thickness, the labeling (BrdUrd) index of epidermal cells or epidermal ODC activity, indices that are used as early markers of tumor-promoting activity. We therefore conclude that MMW exposure under these experimental conditions does not promote or co-promote tumorigenesis in this well-established animal model of skin carcinogenesis.

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The Animal Care and Use Committee at Brooks AFB approved the experimental protocols. The animals involved in this study were procured, maintained and used in accordance with the Federal Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources–National Research Council. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as official Department of the Air Force, Department of the Army, Department of Defense or US Government position, policy or decision unless so designated by other documentation. Trade names of materials and/or products of commercial or non-government organizations are cited as needed for precision. These citations do not constitute official endorsement or approval of the use of such commercial materials and/or products. Approved for public release; distribution unlimited.

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